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Isolation and Characterization of Endostyle-Specific Genes in the Ascidian *Ciona intestinalis*

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Abstract. The endostyle is a special organ in the pharynx of Urochordata, Cephalochordata, and Cyclostomata. It may have arisen in the common ancestor of these taxa, along with a shift to internal feeding for extracting suspended food from the water. In addition, the endostyle has a functional homology to the vertebrate thyroid gland. The endostyle is therefore one of the structures key to the understanding of the origin and evolution of chordates. In the present study, we isolated and characterized cDNA clones for four endostyle-specific genes, *CiEnds1*, *CiEnds2*, *CiEnds3*, and *CiEnds4*, of the ascidian *Ciona intestinalis*. Although the predicted amino acid sequences of the gene products CiENDS1, CiENDS2, and CiENDS3 showed no similarity to known proteins, their mean hydropathy profiles suggest that they are secretory proteins. In addition, CiENDS3 contained a unique repeat of 10 amino acids [R(QPCI)-(RRPC)I]. *CiEnds1* and *CiEnds2* were expressed in zone 6, a protein-secreting glandular element of the endostyle, and *CiEnds3* was expressed in zone 2, another secretory zone. *CiEnds4*, a cytoplasmic actin gene, was predominantly expressed in zones 3 and 5, which are supporting elements of the endostyle. The amino acid sequences of CiENDS1 and CiENDS2 resembled each other. In addition, they resembled a zone-6-specific gene product (HrENDS2) of another ascidian, *Halocynthia roretzi*. The results suggest that these genes are conserved among ascidian species, and therefore they (as well as *CiEnds3* for the protein with a unique motif) may be useful probes for further analyses of molecular mechanisms involved in endostyle development.

Introduction

The endostyle is a specialized organ in the pharynx of tunicates, cephalochordates, cyclostomates, and certain prosobranchiates (Orton, 1912). The ascidian endostyle forms a trough-shaped structure in the ventral wall of the pharynx which extends from the fore-part of the pharynx to the esophagus (see Figs. 1A and 2D). In 1834, Lister first investigated how food particles in the feeding current are trapped in the pharynx of appendicularians. Fol (1876) found that the food is trapped by a mucous substance produced from the endostyle of tunicates. Thereafter, the ascidian endostyle has been intensively investigated by histological and ultrastructural observations (Olsson, 1963, 1965; Aros and Viragh, 1969; Fujita and Nanba, 1971); by the examination of ^{125}I incorporation (Thorpe *et al.*, 1972; Dunn, 1974); by histochemical detection of thyroperoxidase (Fujita and Sawano, 1979); and by partial purification of thyroperoxidase and its enzyme activity (Dunn, 1980).

It is commonly considered that the endostyle of lower chordates may be a homolog and primitive antecedent of the vertebrate thyroid gland, mainly because the organ incorporates iodine (Barrington, 1957, 1958; Salvatore, 1969). However, the endostyle is a mucus-secreting and food-collecting organ, and the ability to concentrate iodine is restricted to a small region of the organ (Olsson, 1963). The general organization of the ascidian endostyle is depicted in Fig. 1B. The cells of this organ are differentiated into eight or nine strips, or zones, that run parallel to one another in longitudinal orientation. The cells of each zone are highly specialized in morphology and function. The cells of zones 7, 8, and 9, like the thyroid cells of higher vertebrates, have an iodine-concentrating activity. The cells of zones 2, 4, and 6 have numerous secretory granules. These cells are believed to secrete the proteins or mucoprotein related to the digestion of food. The cells of zones 1, 3, and 5 are considered supporting elements and also as elements that might play a role in catching and transporting food.

We are interested in molecular developmental mecha-

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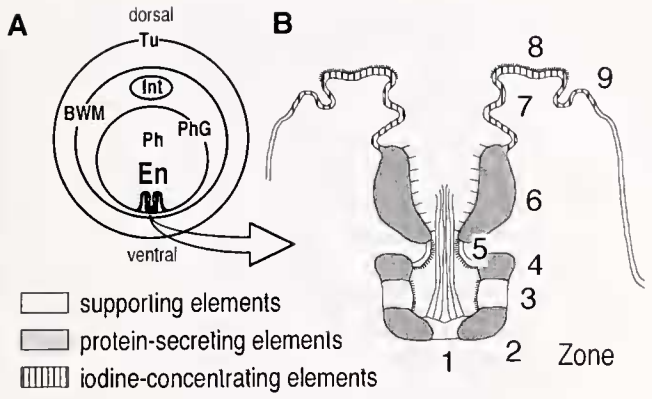

Figure 1. Diagram of the ascidian endostyle. (A) Transverse section of the adult body showing the position of the endostyle (En) in the ventral wall of the pharynx (Ph). BWM, body wall muscle; Int, intestine; PhG, pharyngeal gill; Tu, tunic. (B) Enlargement of the endostyle showing compositional elements or zones of the endostyle. Zones 1, 3, and 5 are supporting elements; zones 2, 4, and 6 are protein-secreting glandular elements; and zones 7, 8, and 9 are iodine-concentrating elements, equivalent to the thyroid gland of vertebrates. [Based on descriptions of Barrington (1957), Thorpe *et al.* (1972), Fujita and Nanba (1971), and Dunn (1974)].

nisms that permitted or accelerated the advent of chordates. The phylum Chordata consists of the subphyla Urochordata (tunicates), Cephalochordata (amphioxus), and Vertebrata. Chordates are categorized as deuterostomes, along with two other invertebrate groups, echinoderms and hemichordates, as was supported by molecular phylogenic studies (Wada and Satoh, 1994; Turbeville *et al.*, 1994). Chordates share several characteristic features including a notochord, a dorsal hollow nerve cord, and pharyngeal gill slits. In addition, lower chordates (including tunicates, amphioxus, and lampreys) share an endostyle. We have emphasized that these hallmarks of the chordate body plan apparently evolved with the emergence of creatures resembling tadpole larvae (Satoh, 1995; Satoh and Jeffery, 1995). Therefore, investigations of the organization of these structures are of salient importance in attempts to understand the origin of chordates.

Coincidently with this change in the mode of larval locomotion, most of the primitive chordates or chordate ancestors may have shifted their feeding system to the use of pharyngeal gill slits for extracting suspended food from the water and of an endostyle for secreting mucus to catch the food particles (*e.g.*, Brusca and Brusca, 1990). This possibility suggests that, in addition to the notochord and nerve cord, the pharyngeal gill and endostyle are key organs that can be used to explore molecular mechanisms involved in the emergence of chordates. In other words, we believe that the origin and evolution of chordates may be approached by isolating genes specific to the pharyngeal gill or endostyle and by analyzing how these genes are organized during evolution in various deuterostomes. In previous studies, we isolated cDNA clones for pharyngeal gill-specific genes (*HrPhG1* and *HrPhG2;* Tanaka *et al.*, 1996) and for endostyle-specific genes (*HrEnds1* and *HrEnds2;* Ogasawara *et al.*, 1996) from the ascidian *Halocynthia roretzi*, which belongs to the order Pleurogona. Both endostyle-specific genes are expressed in zone 6 and encode secreted proteins. In the present study, we attempted the isolation of cDNA clones for endostyle-specific genes from *Ciona intestinalis*, which belongs to the order Enterogona. If both ascidian species conserve endostyle-specific structural genes, they may serve as models for the investigation of the molecular mechanisms involved in the organization of other chordate groups.

Materials and Methods

Biological materials

Adults of *Ciona intestinalis*, *C. savignyi*, and *Styela clava* were collected near the Marine BioSource Education Center of Tohoku University, Onagawa, Miyagi and Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan. After the dissection of adult specimens, tissues and organs were quickly frozen in liquid nitrogen, and kept at −80°C until use.

Isolation of RNAs and construction of cDNA libraries

Total RNA was extracted from the endostyle and pharyngeal gill of *C. intestinalis* by the AGPC method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified with oligotex dT30 beads (Roche Japan, Tokyo). Complementary DNA was synthesized and cDNA libraries were constructed as described in a previous report (Ogasawara *et al.*, 1996). An endostyle cDNA library was constructed using a uni-ZAP-II vector (Stratagene, La Jolla, CA).

Isolation and sequencing of cDNA clones for endostyle-specific genes

The endostyle cDNA libraries were screened differentially. Duplicate filters of the library were made; one was hybridized with a [^{32}P]-labeled total cDNA probe prepared from 5 μg of poly(A)$^+$ RNA of endostyle under high-stringency conditions, and the other was hybridized with a [^{32}P]-labeled total cDNA probe of pharyngeal gill under the same conditions. Plaques that showed positive hybridization with the endostyle probe but were negative for the pharyngeal-gill probe were selected and isolated by two rounds of screening. The specificity of the clones positive for the endostyle was confirmed by a Northern blot analysis. The clones were prepared for sequencing by controlled nested deletion from either the T3 or T7

side and sequenced using the ABI PRISM 377 DNA Sequencer (Perkin Elmer, Norwalk, CT).

Northern blot analysis

The Northern blot hybridization was carried out by the standard procedure (Sambrook *et al.*, 1989), and the filters were washed under high-stringency conditions. DNA probes for blot hybridizations were labeled with [^{32}P]-dCTP using a random primed labeling kit (Boehringer Mannheim, Heidelberg, Germany).

In situ *hybridization*

Juveniles of *C. intestinalis*, *C. savignyi*, and *Styela clava* were fixed in 4% paraformaldehyde in 0.5 *M* NaCl, 0.1 *M* MOPS buffer at 4°C for 12 h. Probes were synthesized by following the instructions from the supplier of the kit (DIG RNA Labeling kit; Boehringer Mannheim). The *in situ* hybridization of whole-mount specimens was carried out basically as described previously (Ogasawara *et al.*, 1996). For the *in situ* hybridization of sectioned specimens, samples were dehydrated with a graded series of alcohol, embedded in polyester wax (BDH), and sectioned at 6 μm.

Results

As in the case of the isolation of cDNA clones for the endostyle-specific genes of *H. roretzi* (Ogasawara *et al.*, 1996), differential screenings of a *C. intestinalis* endostyle cDNA library with total cDNA probes for the endostyle and pharyngeal gill yielded several cDNA clones specific to or enriched in the endostyle library. The preliminary *in situ* hybridization analysis of sectioned specimens demonstrated that transcripts of four cDNA clones were specific to the endostyle. We named the corresponding genes *CiEnds1* (*Ciona intestinalis* endostyle gene 1), *CiEnds2*, *CiEnds3*, and *CiEnds4*. During the screening procedures, we noticed that the *CiEnds1* transcript was abundant in the library, representing nearly 10% of the library clones. When 1 μg of poly(A)$^+$ RNA of the endostyle was electrophoresed, we detected the transcript as a band stained with 0.5 μg/ml ethidium bromide (data not shown).

Characterization of cDNA clone for CiEnds1

As shown in Figure 2C, the Northern blot analysis of the *CiEnds1* transcript in various tissues and organs of a *C. intestinalis* adult detected the transcript of about 2.3 kb only in the endostyle. Hybridization signals were not detected in the pharyngeal gill, body-wall muscle, intestine, or gonad.

The nucleotide sequence of the cDNA clone for *CiEnds1* will appear under the accession number of AB010895 in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The insert of the cDNA clone consisted of 2265 nucleotides, including 17 adenyl residues at the 3′ end. The clone contained a single open reading frame (ORF) of 1950 nucleotides, which predicted a polypeptide of 650 amino acids (Fig. 2A). The calculated molecular mass (Mr) of the *CiEnds1*-encoded protein (CiENDS1) was 75.5 k.

CiENDS1 did not show any sequence motifs shared by transcriptional factors, a transmembrane domain, nuclear localization signals, or motifs found in growth factor proteins. However, as shown in Figure 2B, the mean hydropathy profiles of CiENDS1 showed that the N-terminus was highly hydrophobic. This region had a typical signal peptide sequence that consisted of a positively charged residue (amino acid position 2; K, Lys), a hydrophobic (3–13) region of 10–15 residues, a charged residue (position 15; S, Ser), and a residue containing the short side chain (position 16; A, Ala). A predicted cleavage site of the signal peptide was evident behind the Ala (position 16). This sequence motif strongly suggests that CiENDS1 is a secretory protein, with a probability of 82% determined by using the PSORT Program (Online. PSORT World Wide Web Server: Available: http://psort.nibb.ac.jp). In addition, CiENDS1 contained four putative *N*-linked glycosylation sites (Fig. 2A).

The *in situ* hybridization of whole-mount specimens demonstrated that the signals were restricted to the endostyle (Fig. 2D). In addition, the *in situ* hybridization of sectioned specimens demonstrated that the signals were not distributed over the entire regions of the endostyle but rather were restricted to zone 6 (Fig. 2E). No signals above background level were found in the control specimen hybridized with the sense probe (data not shown).

Characterization of cDNA clone for CiEnds2

The nucleotide sequence of cDNA clone for *CiEnds2* will appear under the accession number of AB010896 in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The insert of the cDNA clone consisted of 2107 nucleotides, including 25 adenyl residues at the 3′ end. The occurrence of a 2.3-kb-long *CiEnds2* transcript only in the endostyle (Fig. 3C) suggested that the cDNA was close to full-length. The clone contained a single ORF of 1950 nucleotides, which also predicted a polypeptide of 650 amino acids (Fig. 3A). The calculated Mr of the *CiEnds2*-encoded protein (CiENDS2) was 77.3 k.

CiENDS2 may also be a secretory protein. As shown in Figure 3B, the mean hydropathy profiles of CiENDS2 showed that the N-terminus was highly hydrophobic. This region had a typical signal peptide sequence that consisted of a positively charged residue (amino acid position 2; K, Lys), a hydrophobic (3–13) region of 10–15 residues, a charged residue (position 15; N, Asp), and a residue

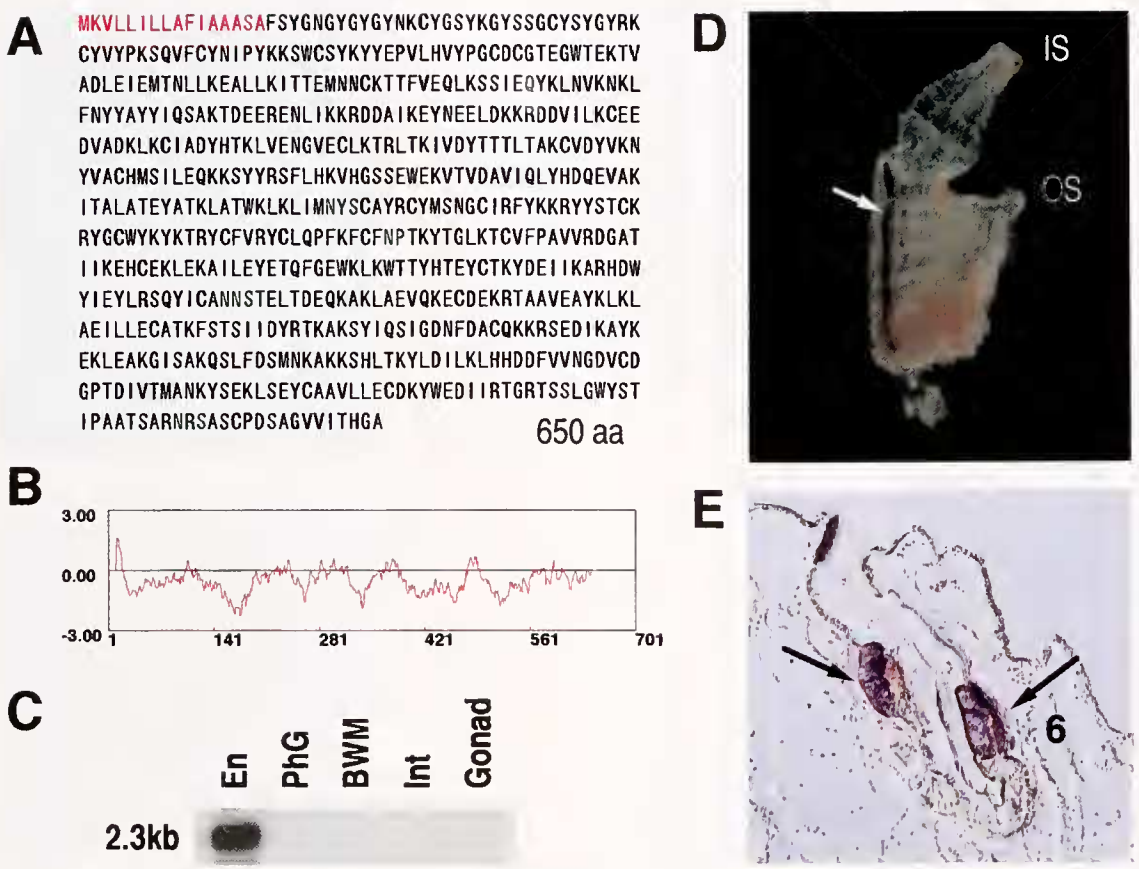

Figure 2. Characterization of the *CiEnds1* gene. (A) The predicted sequence of 650 amino acids of CiENDS1. The predicted signal peptide sequence is shown by red capitals, and putative N-linked glycosylation sites by green capitals. The nucleotide sequence for *CiEnds1* will appear under the accession number of AB010895 in the DDBJ, EMBL, and GenBank nucleotide sequence databases. (B) Mean hydropathy index of the CiENDS1 calculated across a window of 19 residues according to the method of Kyte and Doolittle (1982). The N-terminus of the protein is characterized by a 16-amino-acid-long hydrophobic region that contains the predicted signal peptide sequence (see text for details). This suggests that CiENDS1 is a secretory protein. (C) Distribution of *CiEnds1* transcript in tissues and organs of the adult. Northern blots of poly(A)$^+$ RNA prepared from the endostyle (En), pharyngeal gill (PhG), body-wall muscle (BWM), intestine (Int), and gonad (Gonad) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. The *CiEnds1* transcript of about 2.3 kb in length was detected only in the endostyle. Each lane was loaded with 8 μg of poly(A)$^+$ RNA. (D, E) Localization of *CiEnds1* transcript, as revealed by *in situ* hybridization. IS, incurrent siphon; OS, outcurrent siphon. (D) A whole-mount specimen of a 1-month-old young adult and (E) a cross-section of an adult showing that the signal is restricted to the endostyle (D, arrow) and to zone 6 of the endostyle (E, arrows).

containing the short side chain (position 16; A, Ala). A predicted cleavage site of the signal peptide was evident behind the Ala (position 16). However, the results of the PSORT Program calculation showed that CiENDS2 seems to have an uncleaved N-terminal signal sequence. In addition, CiENDS2 contained a putative *N*-linked glycosylation site (Fig. 3A). CiENDS2 showed 48.3% identity at the amino acid level with CiENDS1, a finding that will be discussed later.

The *in situ* hybridization of whole-mount specimens demonstrated that the signals were restricted to the endostyle (Fig. 3D), whereas that for sectioned specimens demonstrated that the signal was restricted to zone 6 (Fig. 3E). No signals above the background level were found in the other zones.

Characterization of cDNA clone for CiEnds3

The nucleotide sequence of cDNA clone for *CiEnds3* will appear under the accession number of AB010897 in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The insert of the *CiEnds3* cDNA was

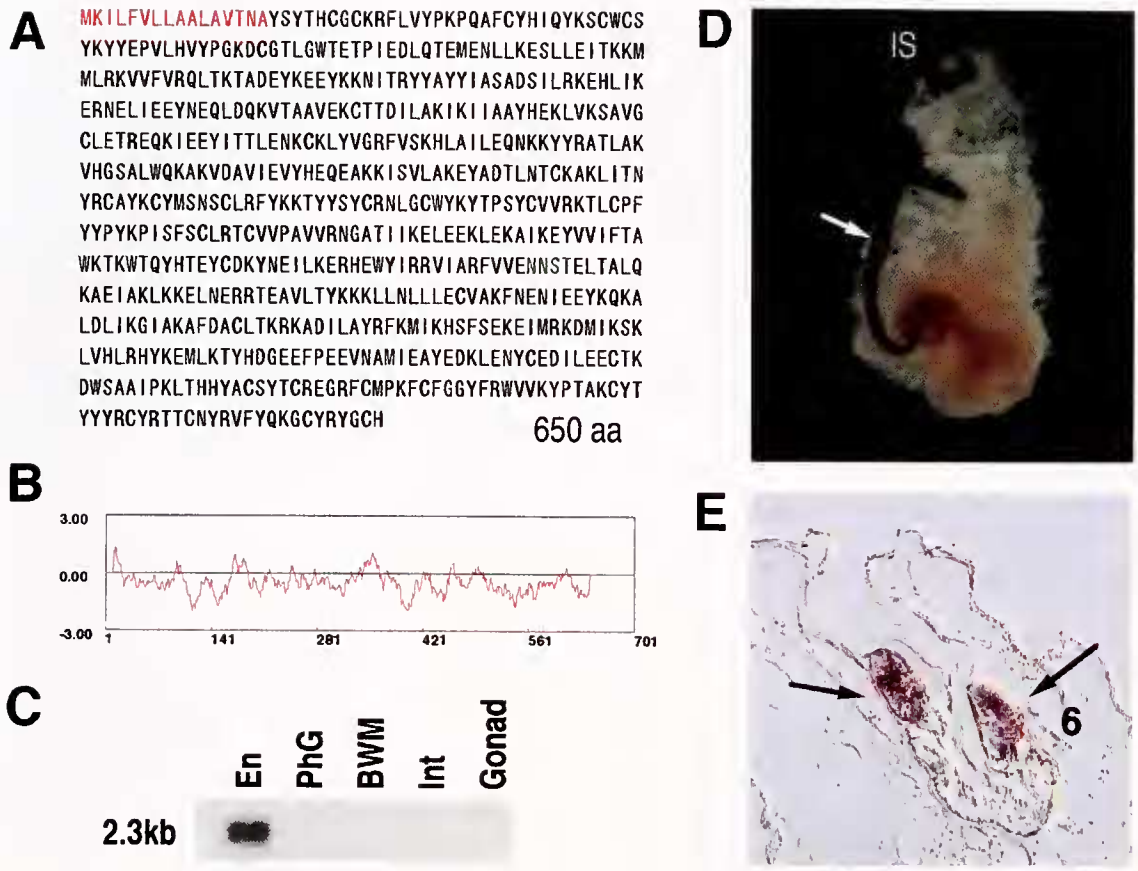

Figure 3. Characterization of the *CiEnds2* gene. (A) The predicted sequence of 650 amino acids of CiENDS2. The predicted signal peptide sequence is shown by red capitals, and a putative N-linked glycosylation site by green capitals. The nucleotide sequence for *CiEnds2* will appear under the accession number of AB010896 in the DDBJ, EMBL, and GenBank nucleotide sequence databases. (B) Mean hydropathy index of the CiENDS2, calculated as in the case of CiENDS1. (C) Distribution of *CiEnds2* transcript in tissues and organs of the adult. The *CiEnds2* transcript of about 2.3 kb in length was detected only in the endostyle. Each lane was loaded with 8 μg of poly(A)$^+$ RNA. (D, E) Localization of *CiEnds2* transcript, as revealed by *in situ* hybridization. IS, incurrent siphon. (D) A whole-mount specimen of a 1-month-old young adult and (E) a cross-section of an adult showing that the signal is restricted to the endostyle (D, arrow) and to zone 6 of the endostyle (E, arrows).

1319 nucleotides, including 17 adenyl residues at the 3′ end. The Northern blot analysis demonstrated the occurrence of a 1.4-kb long *CiEnds3* transcript, which was detected only in the endostyle (Fig. 4C). This suggested that the cDNA was close to full-length. The clone contained a single ORF of 945 nucleotides, which predicted a polypeptide of 315 amino acids (Fig. 4A). The calculated Mr of the *CiEnds3*-encoded protein (CiENDS3) was 35.3 k.

CiENDS3 may also be a secretory protein. As shown in Figure 4B, the mean hydropathy profiles of CiENDS3 showed that the N-terminus was highly hydrophobic. This region had a typical signal peptide sequence that consisted of a positively charged residue (amino acid position 2; R), a hydrophobic (3–13) region of 10–15 residues, a charged residue (position 15; T), and a residue containing the short side chain (position 16; C). A predicted cleavage site of the signal peptide was evident behind the Cys (position 16). This sequence motif strongly suggests that CiENDS3 is a secretory protein, with a probability of 58% determined by the PSORT Program.

In addition, CiENDS3 contained a unique repeat of 10 amino acids (Fig. 4A). The repeat consisted of R(QPCI)(RRPC)I. This type of repeat has not been reported to date in the PDB, SWISSPROT, and PIR databases surveyed.

The *in situ* hybridization of whole-mount specimens demonstrated that the signals were restricted to the endostyle (Fig. 4D). In addition, the *in situ* hybridization of sectioned specimens demonstrated that the signal was restricted to zone 2 (Fig. 4E). No signals above the background level were found in the other zones.

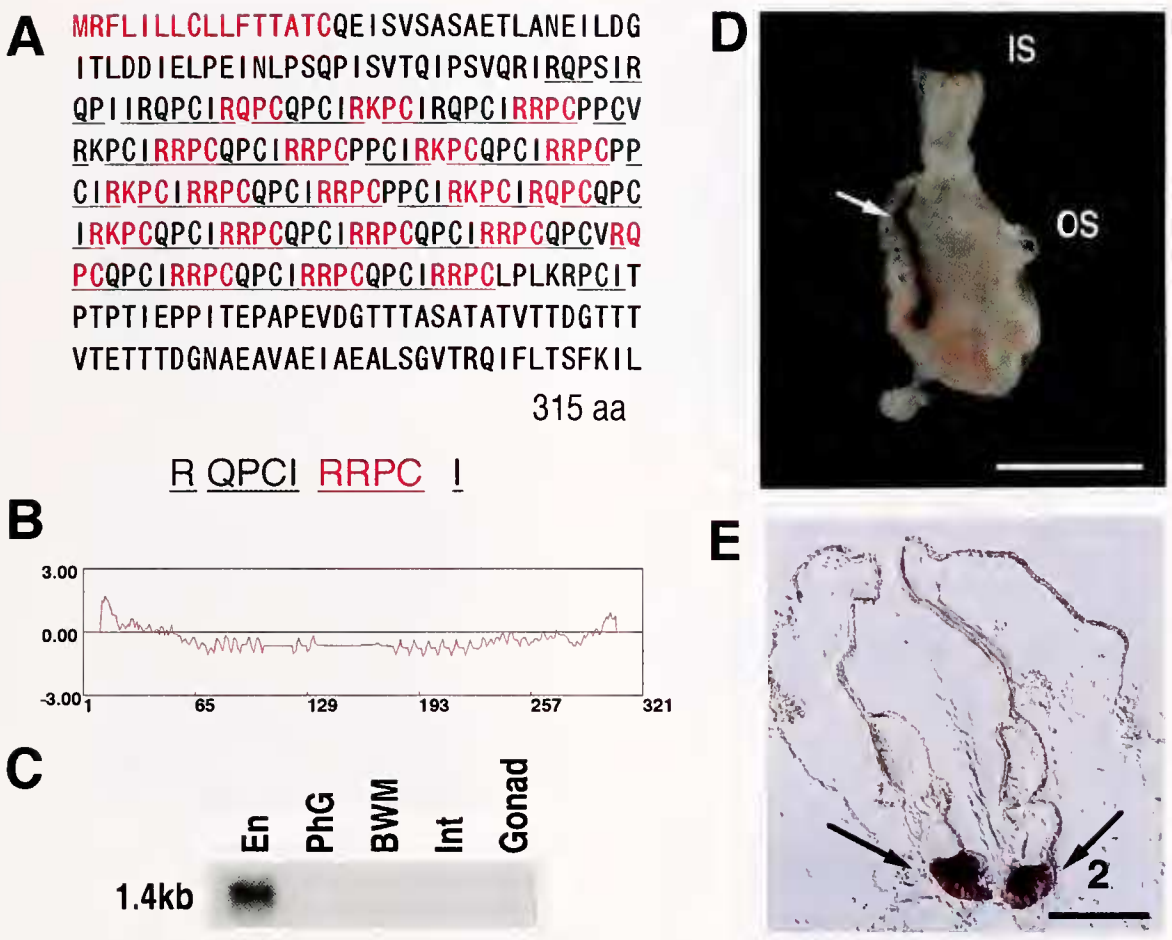

Figure 4. Characterization of the *CiEnds3* gene. (A) The predicted sequence of 315 amino acids of CiENDS3. The predicted signal peptide sequence is shown by red capitals. The sequence contained a characteristic repeat [R(QPCI)(RRPC)I]. The underlined amino acids are identical to those of the consensus sequences. The nucleotide sequence for *CiEnds3* will appear under the accession number of AB010897 in the DDBJ, EMBL, and GenBank nucleotide sequence databases. (B) Mean hydropathy index of the CiENDS3 calculated as in the case of CiENDS1. (C) Distribution of *CiEnds3* transcript in tissues and organs of the adult. Northern blots of poly(A)$^+$ RNA prepared from the endostyle (En), pharyngeal gill (PhG), body-wall muscle (BWM), intestine (Int), and gonad (Gonad) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. The *CiEnds3* transcript of about 1.4 kb in length was detected only in the endostyle. Each lane was loaded with 8 μg of poly(A)$^+$ RNA. (D, E) Localization of *CiEnds3* transcripts, as revealed by *in situ* hybridization. IS, incurrent siphon; OS, outcurrent siphon. (D) A whole-mount specimen of a 1-month-old young adult (scale bar is 1 mm) and (E) a cross-section of an adult (scale bar is 100 μm) showing that the signal is restricted to the endostyle (D, arrow) and to zone 2 of the endostyle (E, arrows).

Characterization of cDNA clone for CiEnds4

The *in situ* hybridization to isolate endostyle-specific cDNA clones demonstrated that the transcript of another clone (*CiEnds4*) was restricted to the zones 3, 5, and 7 (Fig. 5A). Strong signals were evident in zones 3 and 5, supporting elements of the endostyle; weak signals were also evident in zone 7, a putative iodine-concentrating element (Fig. 5A).

The insert of the *CiEnds4* cDNA was 1541 nucleotides, including 19 adenyl residues in the 3′ end. The clone contained a single ORF of 1125 nucleotides, which predicted a polypeptide of 375 amino acids (data not shown). A cDNA clone for *C. intestinalis* cytoplasmic actin has been isolated and characterized in the laboratory of Dr. Takahito Nishikata of Konan University, Kobe, Japan (pers. comm.). The determination of the partial nucleotide sequence of *CiEnds4* cDNA clone revealed that *CiEnds4* encodes a cytoplasmic actin.

The above-mentioned results suggested that the gene or genes for one or more cytoplasmic actin are actively

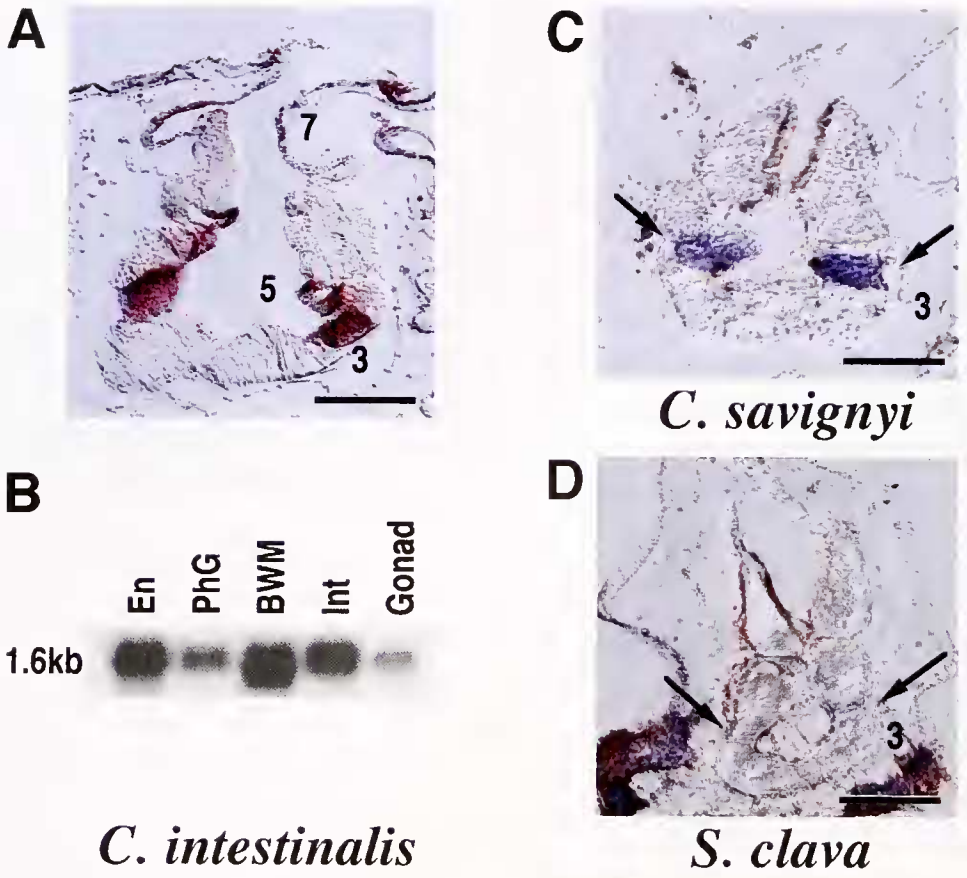

Figure 5. Characterization of the *CiEnds4* gene. The predicted amino acid sequence of CiENDS4 suggested that CiENDS4 is a cytoplasmic actin. (A) Localization of *CiEnds4* transcripts, as revealed by *in situ* hybridization. A cross-section of a young adult showing signals in zones 3, 5, and 7 of the endostyle. (B) Distribution of *CiEnds4* transcript in tissues and organs of the adult. Northern blots of poly(A)$^+$ RNA prepared from the endostyle (En), pharyngeal gill (PhG), body-wall muscle (BWM), intestine (Int), and gonad (Gonad) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. The *CiEnds4* transcript of about 1.6 kb in length, or closely related molecules, was detected in the organs examined. Each lane was loaded with 8 μg of poly(A)$^+$ RNA. (C, D) Cross-reactivity of *CiEnds4* probe with zone 3 (arrows) of the endostyle of (C) *C. savignyi* and (D) *Styela clava*, as revealed by *in situ* hybridization (scale bars in A, C, and D are 100 μm).

expressed in the supporting elements of the endostyle. We therefore examined, using the *CiEnds4* probe, whether the cytoplasmic actin gene is expressed in the supporting elements of the endostyle of two other ascidian species, *C. savignyi* and *Styela clava*. As shown in Figure 5C and D, the *CiEnds4* probe detected cytoplasmic actin transcripts in zone 3 of both species.

In addition, the Northern blot analysis demonstrated the occurrence of a 1.6-kb-long *CiEnds4* transcript not only in the endostyle but also in other organs including the pharyngeal gill, body-wall muscle, intestine, and gonad (Fig. 5B).

Discussion

In the present study, we isolated cDNA clones for four genes (*CiEnds1*, *CiEnds2*, *CiEnds3*, and *CiEnds4*), which are expressed in different zones of the ascidian endostyle. *CiEnds1* and *CiEnds2* are expressed in zone 6 and encode peptides with similar amino acid sequences. *CiEnds3* is expressed in zone 2 and encodes a polypeptide with a novel repeat of 10 amino acids, tentatively called "ends-repeat." *CiEnds4* encodes a cytoplasmic actin that is expressed mainly in zones 3 and 5.

In a previous study, we characterized cDNA clones for two endostyle-specific genes, *HrEnds1* and *HrEnds2*, from the ascidian *H. roretzi* (Ogasawara *et al.*, 1996). Both genes are expressed in zone 6 and encode secreted proteins. Transcripts of both genes are abundant in the endostyle library; each represents about 10% of the cDNA clones of the library. As revealed by the present study, *CiEnds1* is also expressed in zone 6 and encodes a secreted protein. This transcript also represents the most

abundant species in the library. These results strongly suggest that zone 6 plays a major role in the secretion of mucus by the endostyle and has functions different from those of zones 2 and 4. This notion is consistent with a previous ultrastructural observation that the size and structure of the secretory granules in zone 6 differ from those of the other glandular zones 2 and 4 (Aros and Viragh, 1969). Zone 6 occupies the largest area in the endostyle and is characterized as the most developed glandular zone, containing abundant endoplasmic reticulum (Fujita and Nanba, 1971). Aros and Viragh (1969) and Fujita and Nanba (1971) reported that zone 6 contains at least two types of secretory granule—a large electron-dense granule and a smaller granule. Directly facing the pharynx in zone 6 is a wide exit for secretion; in contrast, zones 2 and 4 have only a very limited exit for secretion (Thorpe *et al.*, 1972).

When the amino acid sequences are compared for CiENDS1 and CiENDS2 (Fig. 6) and for CiENDS1, HrENDS2, and CiENDS2 (Fig. 7), these three polypeptides closely resemble each other: the sequence identity was 48.3% between CiEnds1 and CiEnds2, 22.2% between CiENDS1 and HrENDS2 (similarity 43.7%), and 22.8% between CiENDS2 and HrENDS2 (similarity 42.6%). Our previous genomic Southern blotting analysis of *HrEnds2* suggested that there are some other genes in the *H. roretzi* genome that contain a sequence similar to that of *HrEnds2*. In the present study, we isolated two cDNA clones from *C. intestinalis* that contained a sequence similar to that of *HrEnds2*. It is therefore likely that CiENDS1, CiENDS2, and HrENDS2 are members of the same protein family, and it is possible that these genes were derived from a common ancestral gene.

The first aim of our studies is to isolate genes that are expressed in certain zones of secretory function, these genes being common in different species that belong to different orders of ascidians. In the present study, we first attempted the screening of *C. intestinalis* homologs of *HrEnds1* and *HrEnds2* with low-stringency hybridization conditions, using these genes as probes. Unfortunately, we could not isolate any homologs with the conditions we adopted. Therefore, we next tried the differential screening we used in a previous study (Ogasawara *et al.*, 1996), because we thought that if the nature of the endostyle is the same between *C. intestinalis* and *H. roretzi*, we might isolate homologous genes easily using this method. In the present screening, we were able to isolate an endostyle-specific cDNA clone for *CiEnds1;* this clone was highly expressed in zone 6 and contained a sequence similar to that of *HrEnds2*. In the further screening, we isolated *CiEnds2*, which was also expressed in zone 6 and contained sequences similar to that of *HrEnds2*. *Ciends1*, *CiEnds2*, and *HrEnds2* may be related to each other and play an important role in the ascidian endostyle, and therefore they are good candidates for future studies.

Interestingly, the *CiEnds3* encodes a polypeptide with a novel repeat of 10 amino acids [the core repeat is 8 amino acids (QPCI)(RRPC)]. We tentatively call this the "ends-repeat." Because of its uniqueness and conserva-

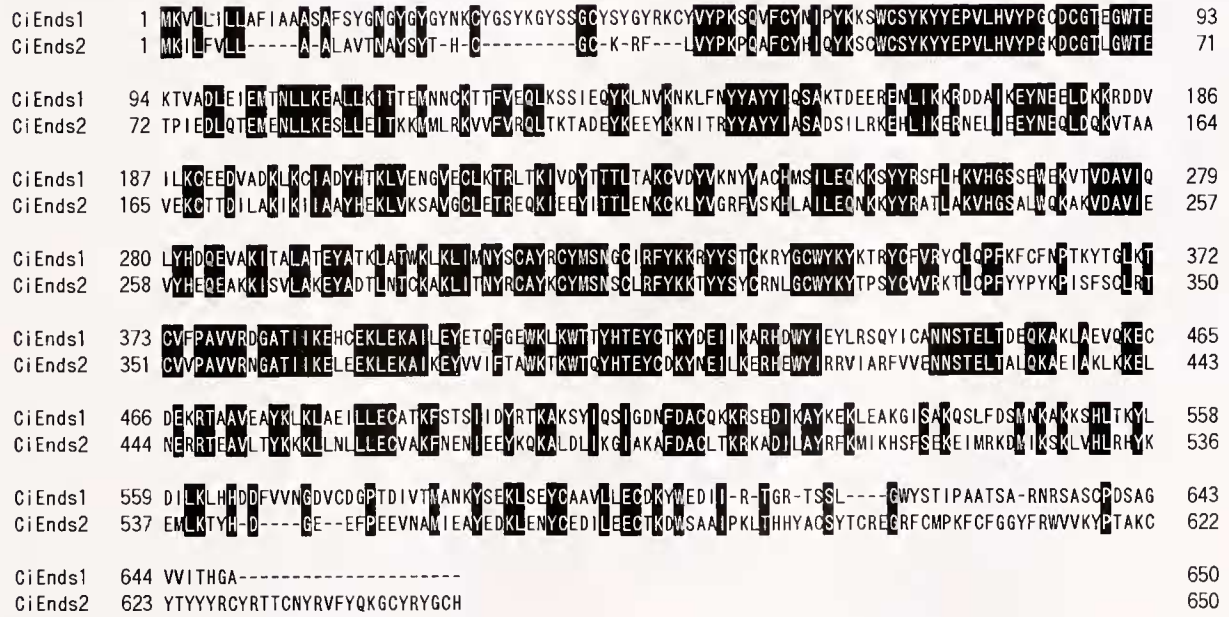

Figure 6. Alignment of the amino acid sequences of CiENDS1 and CiENDS2. Identical amino acids are enclosed by boxes. Gaps were induced to obtain the maximal similarity.

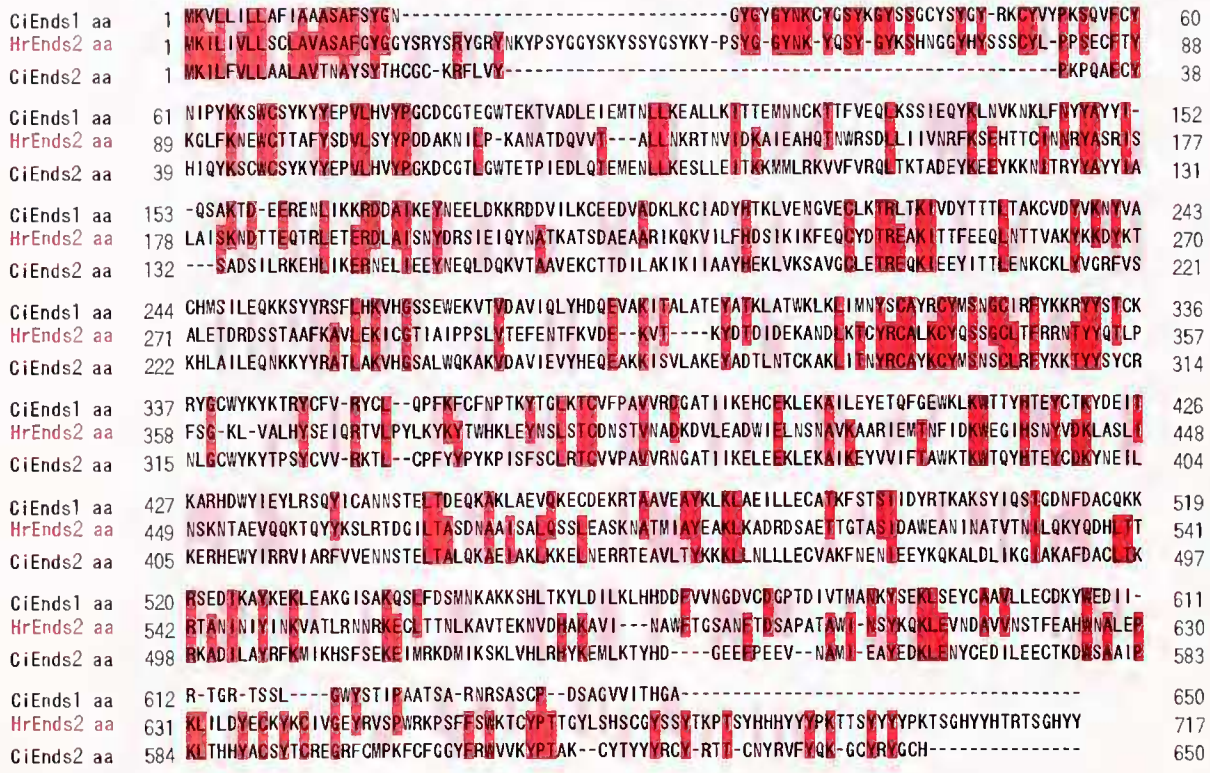

Figure 7. Alignment of the amino acid sequences of CiENDS1, HrENDS2, and CiENDS2. Identical amino acids are enclosed by red boxes, and similar amino acids are enclosed by pink boxes. Gaps were induced to obtain the maximal similarity.

tion among the repeats, this gene is also a good candidate as a probe to explore common mechanisms involved in development and evolution of the endostyle.

There are some differences in the functions of endostyles among ascidian species. The zones that have iodine-concentrating activities (Kobayashi *et al.*, 1983) and thyroperoxidase activities (Dunn, 1974) are different between *H. roretzi, C. intestinalis,* and some other ascidians. However, we think that the functions and gene expressions of the endostyle are basically the same. Indeed, the present study isolated related genes, the nature of which (spatiotemporal expression and amount of the transcript) is the same in *C. intestinalis* and *H. roretzi.* Furthermore, *CiEnds4,* which encoded a cytoplasmic actin, was strongly expressed in zone 3 of *C. intestinalis, C. savignyi,* and *S. clava* (Fig. 5A, C, and D). In future studies, we should use these probes to isolate endostyle-specific genes from cephalochordates, cyclostomes, and hemichordates.

In addition to zones 2, 4, and 6 that secrete mucus, zones 8 and 9 are of special interest because other research has indicated that the vertebrate thyroid gland may be homologous with the endostyle of tunicates (Dunn, 1974; Fujita and Sawano, 1979; Kobayashi *et al.*, 1983), cephalochordates (Tsuneki *et al.*, 1983; Fredriksson *et al.*, 1985; Ericson *et al.*, 1985), and larval lampreys (Egeberg, 1965; Fujita and Honma, 1969). In this and previous studies, we were not able to isolate cDNA clones for genes expressed in zones 8 and 9. The isolation of cDNA clones for genes specific to the thyroid-equivalent elements is very important for elucidating the evolutionary relationship of these elements among ascidians. In addition, the isolation and characterization of genes that are involved in the structure and function of vertebrate thyroid glands are of particular interest for future studies.

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